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In cancer cells, growth factor independence is correlated with overexpression of receptors such as erbB2 in breast cancers. Potential links between tumor-associated overexpression of erbB2 and reduced survival of primary breast cancer patients with metastatic axillary lymph node involvement exist. Hence, growth receptors such as erbB2 make attractive therapeutic targets. We are selectively modifying the physiology of the budding yeast *S. cerevisiae* to speed the identification and study of ligand/receptor interactions - in particular that of erbB2. In brief, we are exploiting the biological process of protein folding in the ER in a completely innovative way so as to achieve these goals. We are taking advantage of this to design a system to search for novel ligands of mammalian receptors, in particular for erbB2. In addition, we are developing methods to identify peptide aptamers that can act as artificial ligands or inhibitors or erbB2 as well as other relevant molecular targets.

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FOREWORD

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Paula Silver 8/27/99

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Annual Report

Principal Investigator: Pamela A. Silver

Project Title: The Identification of Novel Ligands for Cell Surface Receptors

INTRODUCTION (Taken from original proposal)

Purpose and Scope of the Research Program

A general problem in metazoan biology is the identification of the specific ligands for transmembrane receptors. 'Orphan receptors' are becoming more numerous as more DNA sequence becomes available. The funded research focuses on novel approaches for the identification of ligands for cell surface receptors involved in growth control.

In cancer cells, growth factor independence is sometimes correlated with overexpression of growth factor receptors such as erbB2 in breast cancers (19). Overexpression of erbB2 has been shown to activate the ras/MAP kinase pathway, and inhibition of the activation of this pathway has been shown to correlate with decreased cellular proliferation (3, 9). Potential links between tumor-associated overexpression of erbB2 and reduced survival of primary breast cancer patients with metastatic axillary lymph node involvement exist (1, 2, 23). Hence, growth receptors such as erbB2 make attractive therapeutic targets.

We are selectively modifying the physiology of the budding yeast *Saccharomyces cerevisiae* to speed the identification and study of ligand/receptor interactions - in particular that of erB2 given its involvement in breast cancer. In brief, we are exploiting the biological process of protein folding in the ER in a completely innovative way so as to achieve these goals. Accumulation of unfolded proteins in the ER induces transcription of factors that help refold proteins (12). This effect is termed the Unfolded Protein Response (UPR). The Ire1 protein of yeast plays a major role in transducing the signal from the ER lumen to the nucleus (5, 14). Ire1 is embedded in the ER membrane and has a cytoplasmic protein kinase domain. In many respects, Ire1 behaves like a cell surface receptor; Ire1 signaling in yeast is very similar to the ligand-receptor interaction seen in higher organisms. Thus, we are taking advantage of this to design a system to search for novel ligands of mammalian receptors, in particular for erbB2. A second approach involves a novel search for small interacting peptide aptamers that could be used as ligand mimics or inhibitors.

Background of Previous Work Relevant to the Research Program

Proteins destined for secretion, various organelles or the plasma membrane - including growth receptors such as erbB2 - first fold and oligomerize in the ER lumen, which maintains a high concentration of chaperones and other folding enzymes. Proper folding and assembly are prerequisites for continuation of protein transit through the ER, into the Golgi and on to the plasma membrane where receptors interact with their proper ligand.

The synthesis of the ER-resident proteins (such as the chaperone BiP which =Kar2 in yeast and protein disulfide isomerase (PDI)) involved in the protein folding and assembly reactions is regulated in response to cellular requirements. When cells are exposed to reagents such as tunicamycin, that inhibit glycosylation, to reducing agents, or to calcium ionophores that deplete ER-calcium stores, induction of several ER-resident proteins occurs at the transcriptional level (8, 12, 18, 20). All of these treatments are thought to cause improper protein folding in the ER. This behavior has been termed the 'unfolded protein response (UPR)' pathway. A signal from the ER lumen is transmitted to the nucleus where transcription is then activated. Potential unfolded protein response elements (UPREs) have now been identified in promoters of at least 6 genes encoding

ER-based enzymes that are induced in response to unfolded proteins (11, 14, 21). One idea is that the transcriptional response occurs because BiP associates with the misfolded proteins and the cell senses a need to produce more chaperones for the ER.

The *IRE1/ERN1* gene in yeast is required for this pathway (5, 14, 16). A similar pathway mediated by homologues of the yeast proteins has recently been defined in mammalian cells. Mutations in *IRE1* cannot activate transcription of *KAR2/BIP* and *PDI1*, which are regulated by UPREs. *IRE1* encodes a transmembrane serine/threonine protein kinase that is located in the ER membrane with its kinase domain in the cytoplasm (or the nuclear interior) (5, 16). It has been proposed that Ire1p acts by analogy to plasma membrane receptors to transmit a 'signal' from the ER lumen to the cytoplasm. Like mammalian growth factor receptors, Ire1 oligomerizes and is phosphorylated in trans in response to accumulation of unfolded proteins in the ER (22). By analogy to growth factor receptors, this results in a signaling cascade causing the activation of transcription factor(s) in the nucleus. Recently, a bZiP transcription factor, Hac1 (17), has been shown to be essential for transcriptional induction of Kar2/BiP and Pdi by Ire1 (6).

Rationale for the Research Plan

For the purposes of our research program, we have usurped the Ire1 signaling pathway by replacing the putative lumenal ligand binding domain with alternate receptor ligand binding domains to produce a chimeric receptor. Fully functional chimeric receptors consisting of extracellular and cytoplasmic domains from different receptor classes are numerous. We predicted that when the appropriate ligand is secreted into the ER lumen, the chimeric receptor will dimerize and activate the UPR signaling pathway, for which a number of rapid assays exist. Thus, the receptor-ligand interaction will occur in the ER lumen which is more analogous to the extracellular milieu where such interactions normally occur. At the same time, the receptor-ligand interaction can be isolated from other receptors and possible 'cross-talk' which can confuse ligand identification. In the long term, our system will provide a rapid genetic screen for ligands for orphan receptors that previously were mainly identified, if at all, by laborious biochemical means.

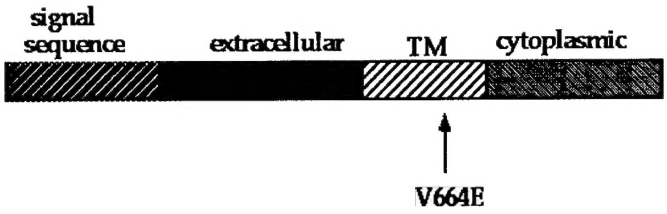
EXPERIMENTAL METHODS AND RESULTS

The goal of the proposed research is to develop a system in *S. cerevisiae* that will allow for the identification of ligands for transmembrane orphan receptors. Specifically, this system will be used to identify a ligand or ligands for the orphan receptor erbB-2 in order to elucidate the role of this receptor in normal and uncontrolled growth. Our approach is to usurp the unfolded protein response pathway of yeast by making chimeric receptors containing the extracellular domain of the orphan receptor fused to the signaling domain of the Ire1 protein and screening for ligands that activate the receptor and in result turn on the unfolded protein response. Initial work focused on using the well characterized Epidermal Growth Factor Receptor (EGFR) and Epidermal Growth Factor (EGF) receptor/ligand pair as a proof of concept (Year 1). In the most recent funding year (Year 2), we have pursued two research goals. First, we focused on construction of the necessary plasmids for expression of the erbB2 chimeras so that we could commence the ligand screening. Second, we began the characterization of peptide aptamer libraries in yeast.

The following is a summary of the current status of the project. In brief, we have made some progress on Specific Aims 2 and 3 of the original application.

Construction of chimeric receptor plasmids (Aim 2)

Progress has been made on construction of the erbB2 chimeric receptor plasmids for expression of erbB2-Ire1 in yeast as illustrated below (and as summarized in last years' report) - the major difference being that we have incorporated the c-myc epitope after the signal sequence so as to confirm production of full-length chimeric proteins..



In addition, we took advantage of a special mutant erbB2 that contains a single amino acid change in the transmembrane domain (V664E). This single mutation yields a ligand-independently activated erbB2 receptor. Thus, when incorporated into our reporter system, we would expect to get a ligand independent signal when this protein is expressed. This will serve as an additional positive control for our system. A second constitutively active receptor construct was constructed and will serve as another ligand-independent proof-of-concept controls. This construct is made up of the extracellular and transmembrane domains of constitutively active human Fibroblast Growth Factor Receptor 2 mutant C342Y found in Crouzon syndrome fused to the signaling domain of Ire1 as illustrated below. The indicated plasmids are being introduced into yeast reporter strains and expression of the encoded chimeric proteins and of the UPR-LacZ reporter are being monitored as was done for the original EGF-Ire1 constructs reported on last year. These plasmids have been tricky and time-consuming to construct. Thus, progress has been only moderate on this part of the project.

Characterization of aptamer libraries in yeast (Aim 3)

For Aim 3, we proposed to apply a novel approach to identify small interacting peptides that may affect receptor function. This approach takes advantage of the bipartite nature of transcription factors. In such a screen, the yeast serve as a vessel in which the interaction occurs and generate the signal that alerts one to the potential interaction. In its most commonly used form, a protein from any organism can be expressed in yeast (the so-called 'bait') in conjunction with a cDNA library from any organism (the 'fish'). The novel methodology to be used here is designed to allow the rapid examination of the interaction of proteins of interest with a large number of random peptides expressed as 'aptamers' (from *aptos* - "to fit"). The aptamers are synthesized from a library of at least 10^8 plasmids that direct the synthesis of randomly encoded 20-mer peptides within *E. coli* thioredoxin, such that the peptides are displayed as loops that protrude from the surface at the thioredoxin active site; the chimeric peptide-proteins have no thioredoxin activity. The gene encoding each aptamer is fused to an activation domain and a nuclear targeting sequence, and the screen for aptamer binding to the protein of interest is carried out in a manner similar to the standard two-hybrid approach (6,14). Thus, the thioredoxin-aptamer is the 'prey', and the 'bait' will be the extracellular domain of erbB-2. We do not expect that the aptamers will induce receptor dimerization, because they will have been isolated using a monomeric target. Instead, in these experiments we will seek peptides that simply bind to the surface of the ligand-binding domain of erbB-2. Some of these may compete for ligand binding. And it is possible to genetically engineer a dimeric thioredoxin-aptamer, which could cause receptor activation.

Once an initial set of aptamers has been identified, an optimized set can be constructed from an initial sequence by mutating each amino acid in the 20-mer peptide to all other possible amino acids. These experiments will allow us to determine the optimal peptide sequence that will, for example, fit into the ligand binding site of erbB-2. Aptamers identified in an initial screen usually have a binding constant of about 10^{-8}M. Knowledge of the peptide sequence will provide information about the ligand binding site that may be used in

rational drug design. Finally, an understanding of the basis for the effect of the peptide on protein function will allow efficient design of anti-cancer strategies.

Much of our work on this aim, which has been carried out this year, concerns the characterization of the aptamer libraries as follows. To validate the approach, we used as 'bait' the well-characterized Cdk4. Cdk4 is a cyclin-dependent protein kinase that functions immediately upstream of Rb and, as such, is a popular target for anti-tumor drug design. Inhibition of Cdk4 activity could impact on cancer cell growth and/or treatment. Therefore, we sought aptamers that specifically bind to Cdk4. Thus far, we have isolated three aptamers that have relatively low affinity and one with relatively high affinity for Cdk4. The sequences have been determined. The amino acid sequence of the highest affinity peptide aptamer is :

G P Q G L V L G E L L T S L G M N W E N P Q G P

The long-term plan would be to seek tighter binders and to test the ability of the aptamers to inhibit Cdk4 function both in vitro as well as in tumor and normal cells. Thus, we have successfully characterized our aptamer library. In addition, these results have potential implications for anti-tumor therapies. Future experiments will involve novel less well-characterized 'baits' including the extracellular domain of erbB2, as proposed in the original grant.

In a second set of experiments, together with Roger Brent's group at the Molecular Sciences Institute, we have described aptamer derivatives that extend the range of functional manipulations that can be conducted with aptamers. An aptamer with increased affinity for Cdk2 was isolated by mutagenizing an existing low affinity aptamer (thus confirming the utility of this approach). This aptamer was then used as a recognition domain in chimeric proteins that contain other functional moieties. For example, aptamers fused to the catalytic domain of ubiqutin ligase caused the ubiquitination of Cdk2. Anti-Cdk2 aptamers that carried a nuclear localization sequence could transport target proteins into the nucleus. Together, these experiments demonstrate that fusion proteins containing aptamer recognition moieties can be used for specific modification of protein function in vivo including selective degradation and re-targeting to different sites within the cell. These results have broad general significance for therapeutic development and will be applicable in a large number of situations.

CONCLUSIONS

In sum, in this second year of funding, we have made some progress on developing our novel approach to search for ligands for orphan receptors. In addition, we have made significant progress on screening for peptide aptamers and have extended this approach to include other molecules relevant to breast cancer. In the coming year, we hope to bring both of these approaches to fruition. Specifically, we will:

1) Continue to set-up the novel system to search for erbB2 ligands. We will continue with our construction and characterization of the necessary chimeric receptors. We hope to obtain from Cadus a library of genes encoding secreted human proteins in yeast, which has been demonstrated to successfully yield ligands for G-coupled receptors in yeast.

2) Continue the characterization of peptide aptamers. Our approaches will be two-fold. First, in the context of the original proposal, we will search for aptamers that bind the extracellular erbB2 domain. Second, we would like to propose to continue our further characterization of aptamers that interact with other molecules of high relevance to breast cancer. Specifically, we would like to further characterize the Cdk4 aptamers by testing whether or not they inhibit Cdk4 function in mammalian cells. If successful, these experiments would have high significance for the development of specific inhibitors of Cdk4 - a project that a number of pharmaceutical companies are pursuing aggressively with limited success at the moment. If we indeed have a *specific* inhibitor of Cdk4 - this would be an invaluable tool for both further proof of concept experiments and therapeutic design.

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